


Index of Claims 	Application/Control No. 10511990	Applicant(s)/Patent Under Reexamination LOYTER ET AL.
	Examiner Snyder, Stuart W	Art Unit 1648

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
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☐ Claims renumbered in the same order as presented by applicant
 ☐ CPA
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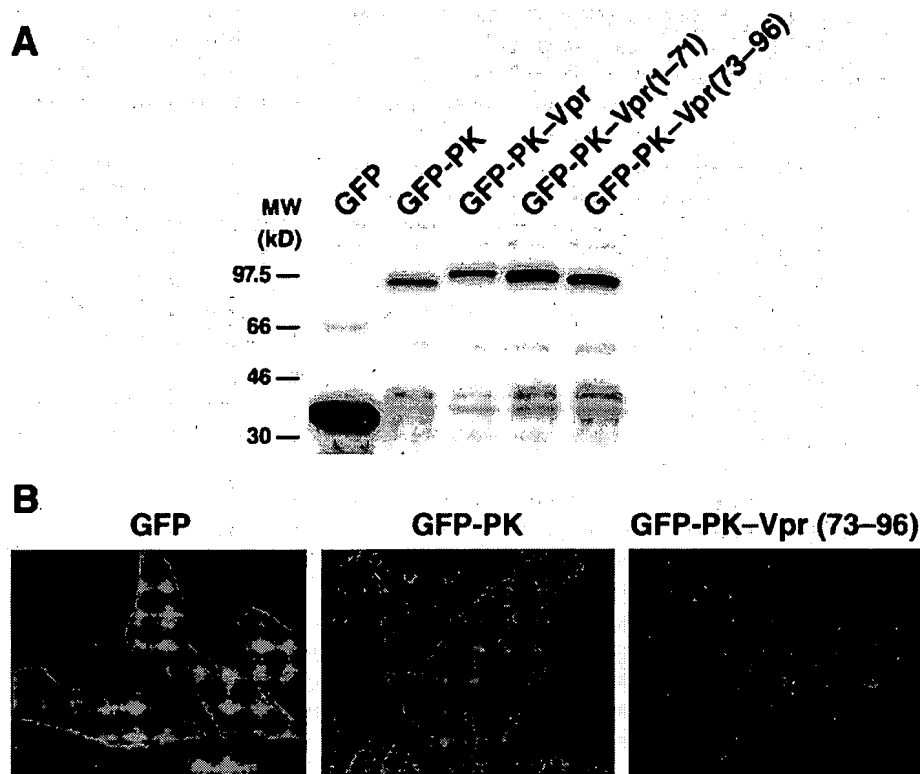


FIG. 1. Characterization of expression vectors encoding GFP, GFP-PK, and GFP-PK fused to Vpr or Vpr fragments. (A) Immunoblotting with an anti-GFP antibody. Note that all of the Vpr-containing chimeras were stably expressed and exhibited an apparent molecular mass that exceeds the passive-diffusion size of the nuclear pore complex. (B) Subcellular localization of the GFP, GFP-PK, and GFP-PK-Vpr(73-96) proteins. Note that while GFP diffuses throughout the cell due to its small size and the GFP-PK protein is cytoplasmic, the GFP-PK-Vpr(73-96) fusion protein is localized principally in the nucleus.

import properties of Vpr, this 14-kDa viral protein (and derivative mutants or Vpr fragments) was fused to the C terminus of a GFP (33 kDa)-PK (55 kDa) chimera to generate an easily monitored large protein complex requiring active transport across the NPC (49). Anti-GFP immunoblotting of lysates prepared from cells transfected with these various expression vectors confirmed the expression of appropriately sized proteins (Fig. 1A). Epifluorescence microscopy of these transfected cells revealed that the GFP protein alone displayed a whole-cell pattern of expression consistent with its small size and passive diffusion throughout the cell (Fig. 1B, left panel). In contrast, the GFP-PK chimera was expressed in the cytoplasm (Fig. 1B, middle panel). Fusion of the Vpr(73-96) fragment to GFP-PK, however, led to an almost exclusively nuclear pattern of localization, confirming the presence of a functional nuclear targeting signal in this Vpr domain.

Alanine-scanning mutagenesis of the entire Vpr(73-96) fragment was then performed to identify residues composing its nuclear targeting signal. Alanines were used to replace the original sequence in sets of three contiguous amino acid substitutions, and in some case, as few as one or two amino acids were changed. Three classes of mutants were produced that had either no effect on nuclear targeting (data not shown), a partial block to nuclear import that led to a whole-cell pattern of protein distribution (Fig. 2B), or composite mutations that disrupted nuclear import altogether (Fig. 2C). The arginines at

amino acid positions 73 and 77, together with the isoleucine at position 74, comprised one part of the import signal, while the four arginines and a single glutamine located between residues 85 and 90 contributed a second part of the nuclear targeting signal. These data thus identify a novel bipartite arginine-rich import motif within the carboxy terminus of Vpr that is able to direct the import of a GFP-PK fusion protein.

Discovery of an NES in the amino-terminal portion of HIV-1 Vpr. We next turned to the analysis of the second Vpr nuclear targeting signal residing in the Vpr(1-71) fragment (26). This portion of Vpr is notable for the presence of two distinct leucine-rich α -helices, both of which have been suggested to play a role in nuclear targeting of Vpr (37, 38, 46, 73). However, previous mutagenesis experiments were performed in the context of the full-length Vpr protein and were thus potentially confounded by the presence of the import signal within the domain from residues 73 to 96. To our surprise, the GFP-PK-Vpr(1-71) or the GFP-PK-Vpr fusion proteins, expressed in either HeLa or 293T cells, localized in the cytoplasmic compartment despite nuclear expression of the control GFP-PK-Vpr(73-96) (Fig. 3A). To address possible abnormalities of fusion protein folding, additional constructs with Vpr positioned at the N terminus of the chimera or separated from the GFP-PK moiety by a glycine spacer were prepared. However, each of these fusion proteins similarly localized to the cytoplasm (data not shown).

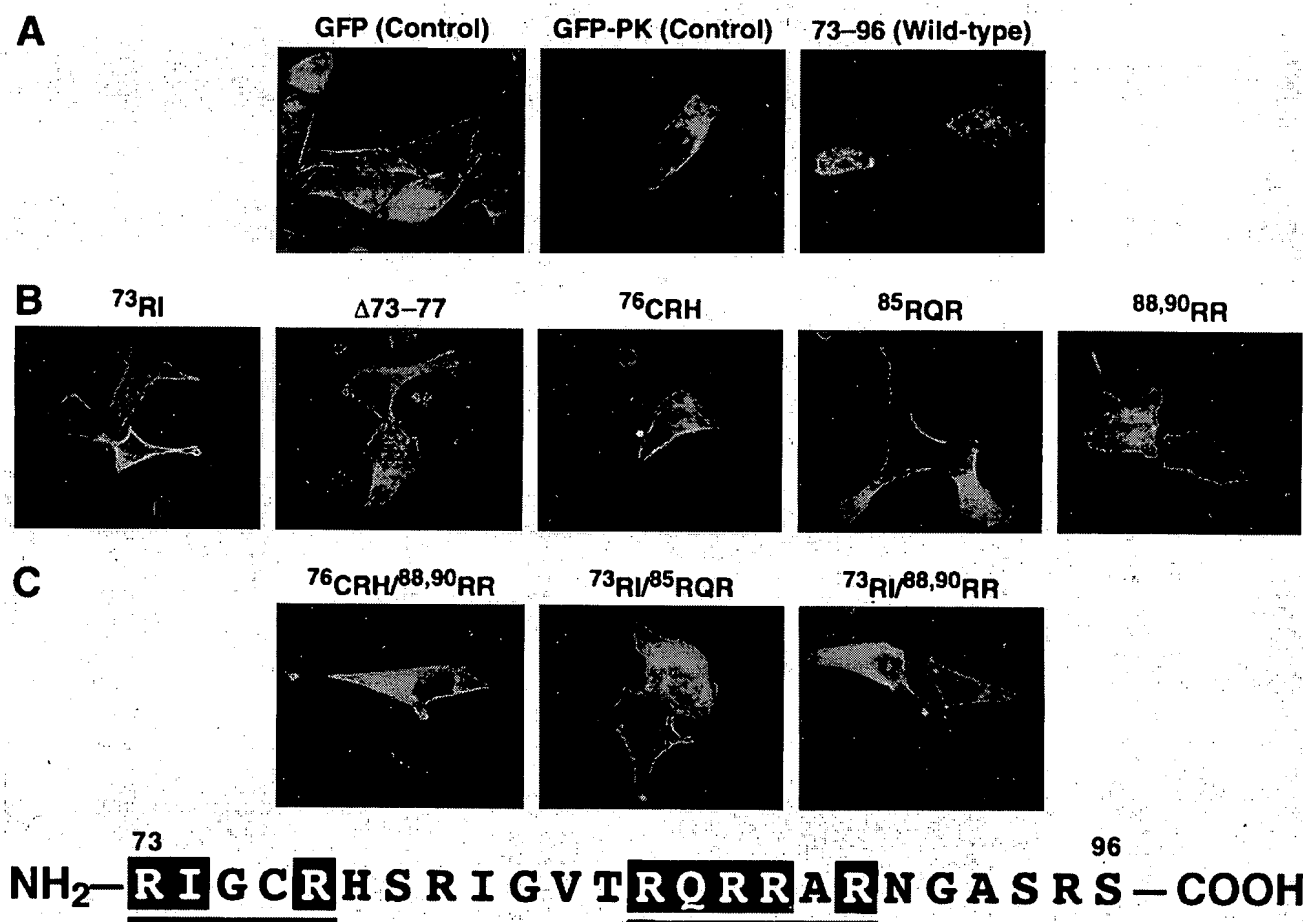


FIG. 2. Identification of specific residues in the amino acid 73 to 96 domain of Vpr required for nuclear import of GFP-PK-Vpr(73-96). Alanine-scanning mutagenesis was performed throughout this 24-amino-acid segment. Note that three phenotypes were obtained, including nuclear (control row A, third panel), whole-cell (B), and cytoplasmic (C) localizations. Mutations that did not disrupt import are not depicted. The cytoplasmic phenotype was obtained only when composite mutants producing a whole-cell pattern of distribution were prepared. Except for the $\Delta 73-77$ deletion mutation, the letters and superscript numbers above each figure correspond to the amino acids in NL4-3 Vpr that were replaced by alanine residues. Note the bipartite arginine-rich nature of the nuclear targeting signal summarized at the bottom of the figure, where key residues are highlighted.

We next considered the possibility that these fusion proteins were shuttling into and out of the nucleus but appeared cytoplasmic due to a longer dwell time in that cellular compartment. This hypothesis was supported by the fact that the leucine-rich α -helical domains resemble the NES recognized by the export protein CRM1. Nuclear export mediated by CRM1 is inhibited in the presence of LMB due to a covalent modification (32). Accordingly, we studied the subcellular localization of GFP-PK-Vpr(1-71) and GFP-PK-Vpr in the presence of graded amounts of LMB and observed a dose-dependent accumulation of both fusion proteins within the nuclei of transfected cells (Fig. 3B). Thus, we have shown that Vpr(1-71) possesses both an NLS and an NES. As an added control, we examined the effect of these same doses of LMB on shuttling of a cyclin B1-GFP fusion protein and found that the same drug concentrations were required to inhibit its nuclear export (data not shown).

Both leucine-rich helical domains participate in formation of a nuclear targeting signal. To further characterize the res-

idues between amino acids 1 and 71 that were involved in Vpr import, we analyzed the import properties of GFP-PK-Vpr(1-71), focusing on specific mutations within the 22 LLEEL 26 and 64 LQQL 68 motifs located at the center of each helix because (i) these leucine-rich domains are highly conserved in HIV-1 Vpr and (ii) if one or both of these motifs are indeed involved in export, there is precedence for an overlapping import signal (41, 42). We took advantage of the fact that LMB could unmask nuclear import by preventing export of the GFP-PK-Vpr(1-71) fusion protein. Mutagenesis of the leucine residues revealed that while the wild-type form of Vpr(1-71) was able to direct nuclear localization in the presence of LMB, complete disruption of either helix by alanine substitutions abolished this phenotype (Fig. 4). More detailed analysis showed that the amino terminal leucines of each helix appear to be required for import (leucines at positions 22, 23, and 64). Further, experiments fusing the GFP-PK protein with subfragments of Vpr, including residues 1 to 31, 25 to 48, and 48 to 71, revealed that neither of these segments were sufficient to me-

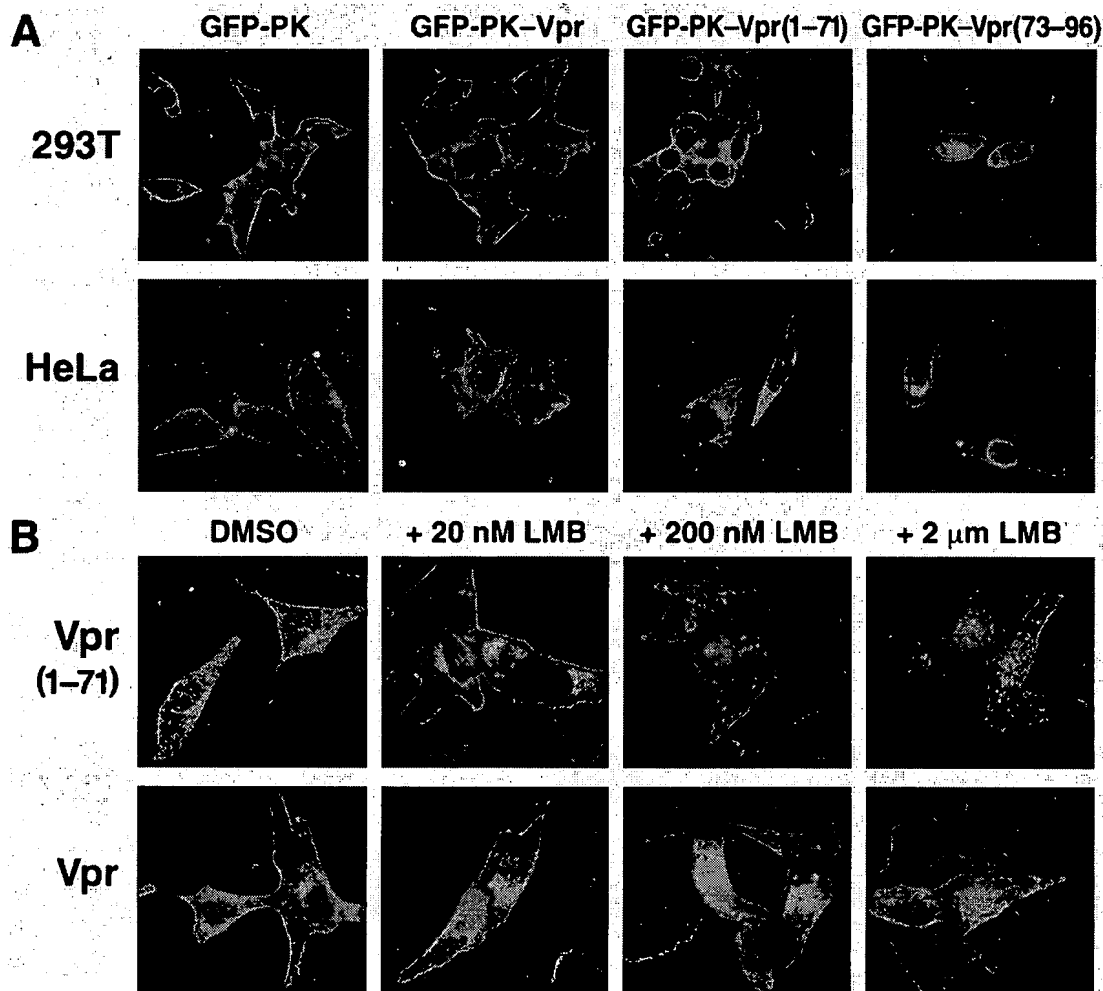


FIG. 3. The Vpr(1-71) domain contains an NLS as well as an LMB-sensitive NES. (A) Each of the indicated chimeras was expressed in either 293T cells or HeLa cells, and subcellular localization of the fusion proteins was assessed by epifluorescence. Note that while the GFP-PK control protein was cytoplasmic and the GFP-PK-Vpr(73-96) chimera was nuclear, the GFP-PK-Vpr(1-71) and even the full-length GFP-PK-Vpr fusion proteins were cytoplasmic. (B) Since Vpr(1-71) contains two leucine-rich domains with homology to NESs recognized by CRM1, the subcellular localization of these cytoplasmic Vpr fusion proteins was studied in the presence of graded doses of LMB, an inhibitor of CRM1. Note that LMB produced dose-dependent accumulation of GFP-PK-Vpr and GFP-PK-Vpr(1-71) in the nucleus.

diate nuclear localization of the GFP-PK chimera in the presence or absence of LMB (data not shown). Together, these data indicate that each leucine-rich helix plays a role in nuclear import of GFP-PK-Vpr(1-71) but that neither alone provides an active NLS. Thus, Vpr contains a novel bipartite, leucine-rich nuclear import signal within the 1 to 71 domain.

Characterization of each nuclear import signal in the context of full-length Vpr demonstrates a cooperative phenotype. We next sought to characterize the two separate import signals in the context of full-length Vpr using the GFP-PK-Vpr fusion protein. It was already clear that despite the presence of two import signals in full-length Vpr, nuclear export of the GFP-PK-Vpr fusion protein predominated (Fig. 3). Therefore, we again employed LMB to unmask nuclear import by blocking nuclear export. First we introduced mutations into Vpr in the arginine-rich import signal that abrogated import in the context of GFP-PK-Vpr(73-96) (Fig. 5A). Disruption of the carboxy-terminal import signal in Vpr in the context of the GFP-

PK-Vpr fusion did not interfere with nuclear import. In contrast, mutation of the leucine-rich helices continued to abrogate nuclear import of intact Vpr (Fig. 5B). However, the presence of the domain from residues 73 to 96 expanded the number of leucine residues required for effective nuclear import. Specifically, in contrast to Vpr(1-71) import, where only leucines 22, 23, and 64 were implicated, replacement of leucines 26 and 68 by alanines altered nuclear import of full-length Vpr fusion proteins. Thus, the presence of the carboxy-terminal, arginine-rich portion of Vpr appears to influence the recognition of the Vpr(1-71) import signal. This implies that the localization of Vpr might be influenced by proteins that bind or obscure one of the import signals.

Identification of the HIV-1 Vpr NES using a modified heterokaryon assay. We next attempted to identify the NES in Vpr using cell fusion experiments known as polykaryon or heterokaryon analyses. In such studies, cells containing the potential shuttling protein of interest in the "donor" nucleus

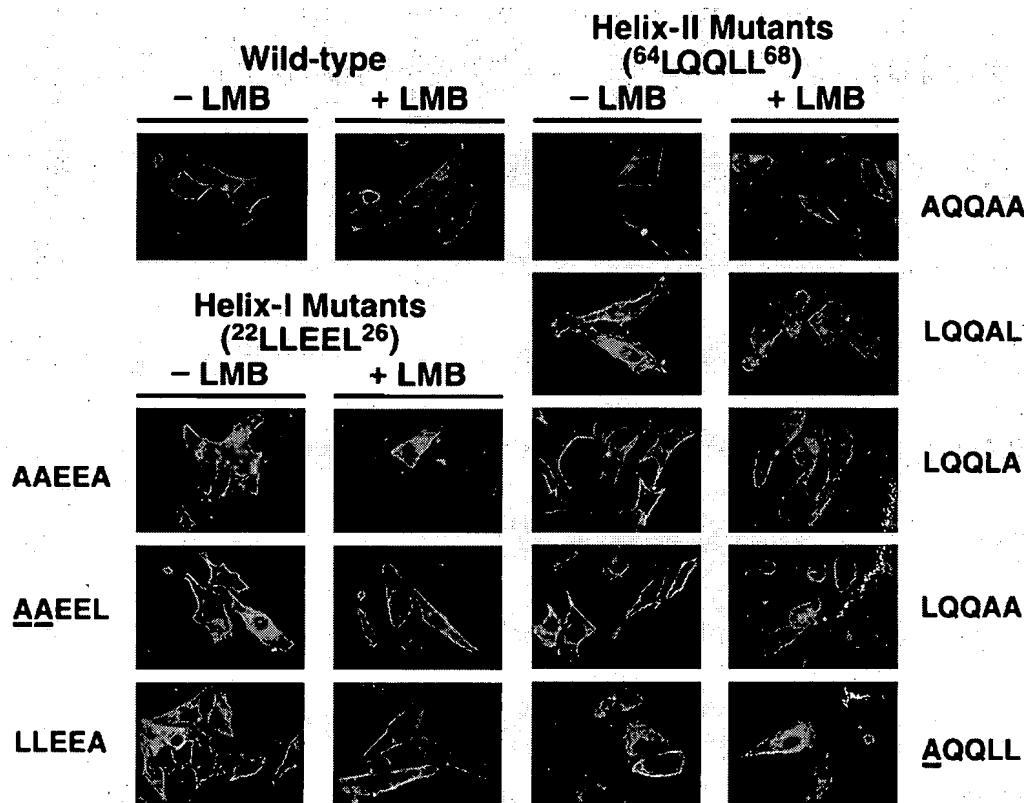


FIG. 4. Nuclear trapping with LMB to map the nuclear import signal in Vpr(1-71). Mutagenesis was focused on the highly conserved leucine residues within helix I (²²LLEEL²⁶) and helix II (⁶⁴LQQL⁶⁸) of the Vpr(1-71) fragment. HeLa cells were transfected with GFP-PK-Vpr(1-71) or the designated mutants and exposed to LMB for 1 h prior to fixation and visualization. Replacement of all three leucines in either helix abolished nuclear import in the presence of LMB. Finer mapping revealed that the first two leucines in helix I and the first leucine in helix II were required for nuclear import, as indicated by bold underlining.

are fused to target cells using PEG, which will then expose recipient nuclei to a common cytoplasmic milieu including any protein exported from the original nucleus. However, this technique requires that the protein of interest be predominantly nuclear, so that if export occurs (if an NES exists), the shuttling protein will be able to enter the cytoplasm of the multicell fusion product and be imported into newly introduced nuclei contained within the heterokaryon. GFP-PK-Vpr clearly shuttled, but it resided predominantly in the cytoplasm of the transfected cell. Therefore, we placed a classical NLS from the SV40 large T antigen at the amino terminus of the fusion protein to see if this would shift the predominant dwell time from the cytoplasm to the nucleus. Indeed, the classical NLS dominated over the export signal in Vpr (Fig. 6A). The question still remained, however, whether this new NLS-GFP-PK-Vpr fusion protein retained the ability to shuttle. We observed that, after overnight transfection, a 28-kDa RFP was located in both the nucleus and the cytoplasm of the donor cells. However, during the 2-h incubation required to generate the polykaryon, the RFP diffused throughout the united cytoplasm yet was excluded from the nontransfected recipient nuclei. This experimental protocol obviated the need to microinject purified proteins, a technique used to distinguish the donor nucleus from potential recipient nuclei. Further, the transfected RFP not only highlighted the untransfected recip-

ient nuclei but also demarcated the boundaries of the newly formed heterokaryons. Thus, this technique became an excellent tool to confirm nuclear shuttling by the GFP-PK-Vpr protein (Fig. 6B). Using this strategy, we further demonstrated that mutation of the leucines at positions 64, 67, and 68 within the second helix, but not the leucines of the first helix, disrupted nuclear shuttling (Fig. 7). These data demonstrate that the NES at least partially overlaps the distal portion of the nuclear import signal in the Vpr(1-71) fragment.

DISCUSSION

A distinguishing feature of the primate lentiviruses is their ability to productively infect nondividing target cells such as terminally differentiated macrophages. HIV-1 encodes three karyophilic proteins, Vpr, matrix, and integrase, that function in a redundant or possibly cooperative manner to promote translocation of the relatively immense viral PIC across the limiting nuclear pore. While matrix and integrase utilize the classical importin α /importin β -dependent pathway of nuclear import, Vpr lacks identifiable canonical NLSs and, moreover, Vpr import is not blocked by inhibitors of the importin α /importin β or M9 pathways (14, 15, 26, 30). Instead, Vpr contains at least two novel import signals, one in the helical, amino-terminal portion of Vpr between amino acids 1 and 71 (26, 30)

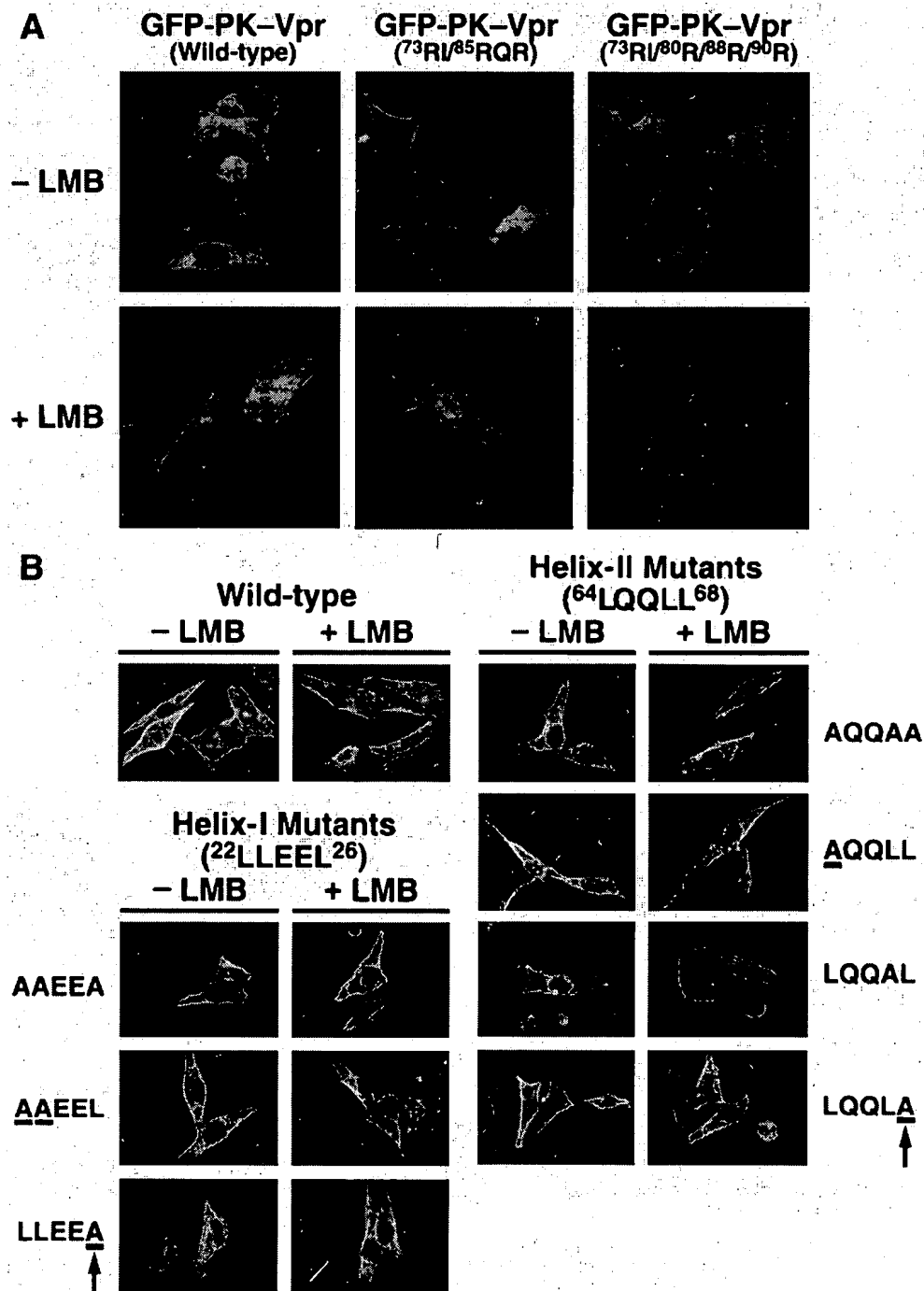


FIG. 5. Assessment of the nuclear localization properties of GFP-PK-Vpr. (A) The nuclear import of GFP-PK-Vpr chimeras containing the disabling composite mutations in the arginine-rich carboxy-terminal signal was studied. Note that the amino-terminal nuclear targeting signal within the amino acid 1 to 71 domain was sufficient to promote nuclear uptake of the chimera containing full-length Vpr in the presence of LMB. (B) The leucine requirement in the amino-terminal helical NLS was assessed for full-length Vpr, as described in the legend to Fig. 4. Note that the arginine-rich nuclear targeting signal did not support nuclear import when all of the leucines in either helical region were replaced by alanines. Further, in the presence of the full-length Vpr, all of the leucines except leucine 67 proved to be requisite for nuclear uptake of the chimera, as indicated by bold underlining. Differences in the leucine dependence between full-length Vpr and Vpr(1-71) are indicated by arrows.

and a second in the loosely folded carboxy-terminal portion between amino acids 73 and 96 (26, 80). Using digitonin-permeabilized HeLa cells to study the nuclear import of recombinant Vpr fused to β -galactosidase, we previously found

that nuclear targeting of Vpr is preserved in the absence of a RanGTP gradient and with limited energy (26). In the present study, we now define and characterize the distinct nuclear targeting signals present in the Vpr(1-71) and Vpr(73-96)

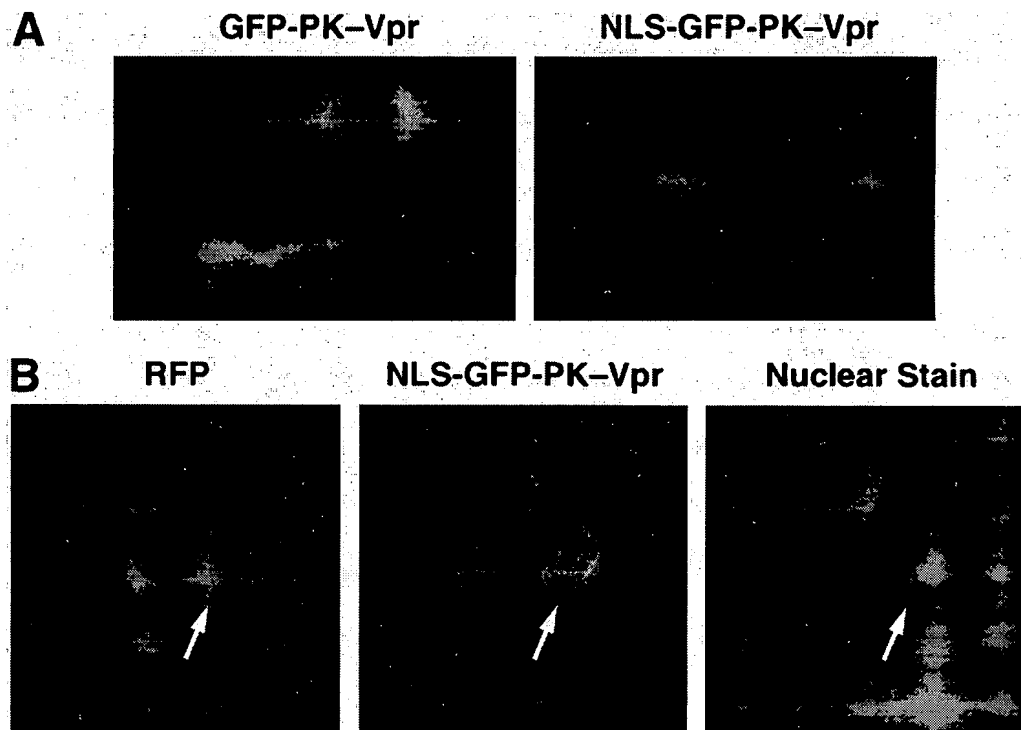


FIG. 6. Heterokaryon analysis to study the NES within Vpr. (A) For these studies, a new chimera was produced incorporating the SV40 large T antigen NLS at the N terminus of the GFP-PK-Vpr fusion protein to promote nuclear predominance. Indeed, the classical NLS was able to overcome the export signal in Vpr and induce nuclear localization. (B) Cotransfection with the 28-kDa RFP was used to mark the boundaries of heterokaryons formed between transfected and nontransfected cells fused with PEG. This fluorescent protein proved a fortuitous choice since it entered the donor nucleus after overnight transfection but failed to diffuse into the nontransfected (recipient) nuclei in the heterokaryons during the time course of these studies. As shown in panel B, the NLS-GFP-PK-Vpr fusion protein effectively shuttled from the red donor nucleus (arrow) into the new nuclei (unstained) of the heterokaryon. Arrows indicate the transfected donor nucleus. Hoechst 33342 staining of all nuclei is shown.

fragments. Specifically, we demonstrate the presence of a bipartite arginine-rich import signal in the carboxy terminus of Vpr and a bipartite leucine-rich signal in the amino-terminal region.

In view of these two functional nuclear targeting signals, our finding that the GFP-PK-Vpr chimera is almost exclusively localized to the cytoplasm was surprising. However, using LMB and heterokaryon analysis, we discovered that Vpr also contains a functional CRM1-dependent NES and participates in nuclear shuttling. Consistent with the ability of LMB to block the binding of outbound cargoes containing a leucine-rich NES through covalent modification of the CRM1 exporter (32), we mapped the NES to a leucine-rich segment in the second α -helical domain in the Vpr(1-71) fragment. Thus, Vpr joins Rev, matrix (9), and Tat (61) as the fourth HIV protein with both nuclear import and export signals. Of note, while the addition of LMB to GFP-PK-Vpr-transfected cells clearly resulted in a nuclear phenotype, this relocalization was not always complete, suggesting the possibility of a CRM1-independent component of Vpr export.

A new class of proteins that contains an overlapping and sometimes inseparable NLS and NES—termed the NS—has recently been recognized (41). While the NES of Vpr clearly involves the distal leucine-rich domain (⁶⁴LQQLL⁶⁸), the full extent of the overlapping import signal is not completely mapped. No subfragment of Vpr that excludes either of the

two leucine-rich helices is sufficient to mediate nuclear import of the GFP-PK fusion protein. However, mutation of the first leucine-rich motif impairs nuclear import without altering nuclear export. A converse mutation that compromises nuclear export without altering nuclear import has not yet been identified. Interestingly, human TAP, a protein that recognizes the constitutive RNA transport element of type D retroviruses and facilitates nuclear export, is a member of the NS family of shuttling proteins (20, 29). While it is unknown whether Vpr delivers a cargo to the cytoplasm during its export, nucleic acid binding by Vpr has been detected (77).

While the import signals present in Vpr are not considered to be canonical, there are a growing number of proteins with the ability to direct import through other recognition sequences. Arginine-rich import signals have been identified in HIV-1 Tat (66) and Rev (31) and in human T-cell leukemia virus Rex (56), cyclin B1 (44), and human TAP (2). Interestingly, all of these proteins contain an NES and are able to shuttle into and out of the nucleus. The arginine-rich NLS binds to importin β directly and bypasses the need for importin α (50, 66). In fact, Tat, Rev, and Rex compete for the importin α binding site on importin β . While previous data indicate that excess importin β binding-domain peptide does not block nuclear import mediated by Vpr(73-96) (26), investigations are under way to determine whether Vpr binds to importin β on a unique determinant or perhaps interacts with a related impor-

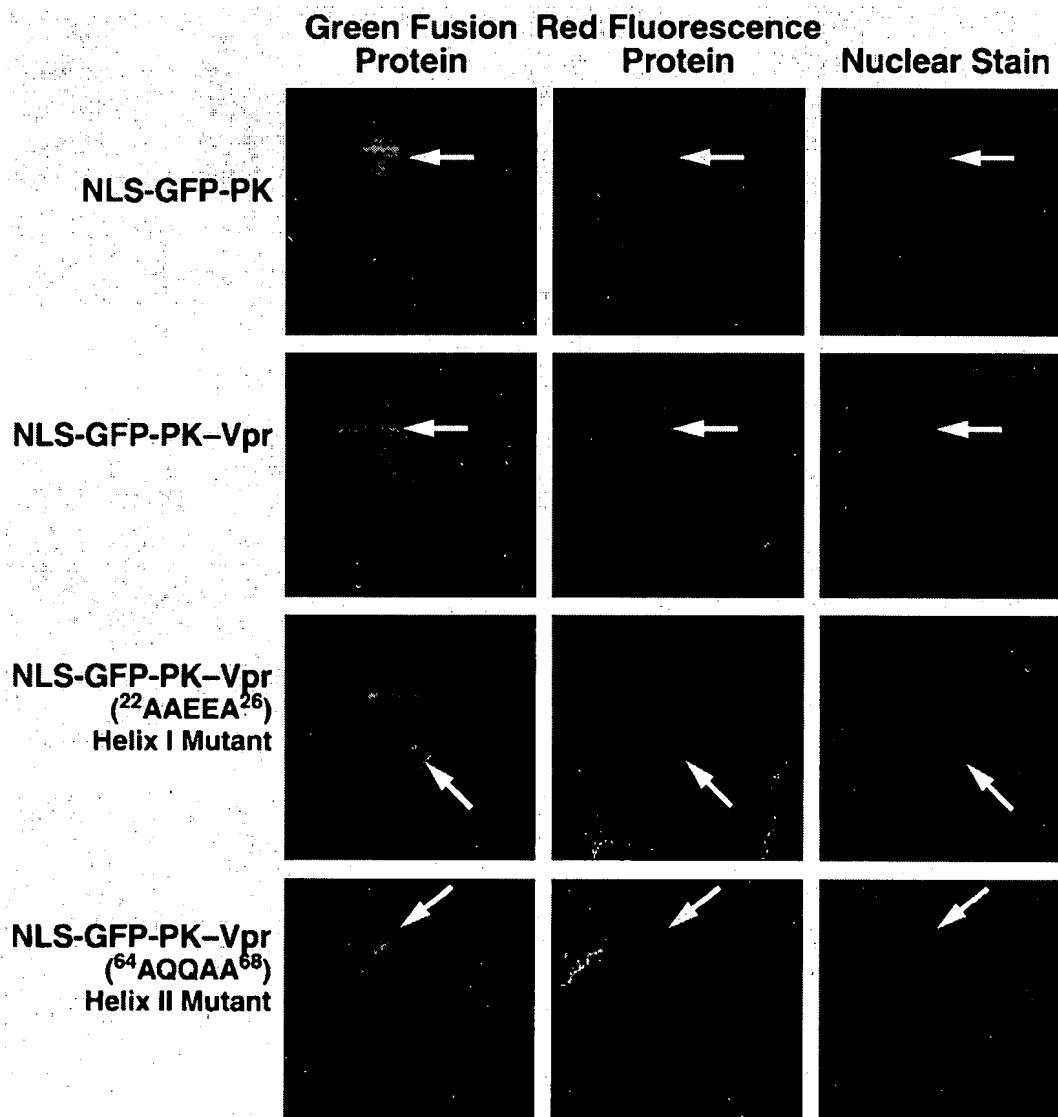


FIG. 7. Mapping of the NES within Vpr. Heterokaryon analyses as described for the experiment in Fig. 6 were performed with the NLS-GFP-PK-Vpr chimeras containing alanine substitutions for each of the three leucines within both of the helical domains. Note that mutation of the leucines in the first helix (²²LLEEL²⁶) had no effect on shuttling, whereas mutation of the leucines in the distal helix (⁶⁴LQQL⁶⁸) abolished shuttling. Thus, the distal leucine-rich domain appears essential for nuclear export. Arrows indicate the transfected donor nucleus. Hoechst 33342 staining was used to display all nuclei.

tin. Of course, Vpr may also function as an importin β homologue through its direct binding to nucleoporins within the NPC (12, 53, 67).

Vpr can interact with heterologous proteins through interactions in both leucine-rich domains (69, 73, 78). Our studies now implicate these leucine-rich domains in nuclear import. The only other example of a leucine-dependent nuclear import signal comes from work showing that a basic helix-loop-helix-leucine zipper motif from the sterol regulatory element binding protein is able to engage importin β directly (45). Whether the leucine motifs in Vpr operate in a similar manner remains to be determined. To our knowledge, this is the first example of a CRM1-dependent NES that overlaps an NLS, and it will

probably prompt reexamination of such NSs in other proteins that lack a classical import signal.

Our studies also provide data regarding Vpr-mediated G₂ cell cycle arrest and the issue of whether nuclear expression of Vpr is required for this response. In previous work, the G₂-arresting property of HIV-1 Vpr has been shown to be dissociated from nuclear import by mutational analyses (10, 37, 65, 67). However, these studies were performed in the context of full-length Vpr, which we now know has the ability to shuttle into and out of the nucleus. Thus, the prior conclusion that cytoplasmic forms of Vpr can induce G₂ cell cycle arrest must be reassessed. We have identified a mutation in the first leucine-rich helical domain of Vpr, where replacement of the

three leucines by alanines at positions 22, 23, and 26 (²²LLEEL²⁶) completely blocks nuclear uptake in the context of a GFP-PK chimera. Experiments utilizing this mutant as a hemagglutinin epitope-tagged version (58) demonstrate that it retains full G₂ cell cycle arresting properties similar to the results of others (37), thus supporting the notion that transport of Vpr across the NPC is not required for its effects on the cell cycle.

What is the role of nuclear shuttling by Vpr? Since there are two nuclear import signals within Vpr and one of these overlaps the NES, it is possible that these import signals act in a sequential or even cooperative manner to ensure effective PIC import in HIV-infected macrophages. This hypothesis is supported by the observation that the arginine-rich import signal influences recognition of the leucine-rich NLS. In terms of the NES, Vpr must be incorporated into newly formed virions to ensure nuclear targeting of these virions into subsequent cellular hosts. The presence of a functional NES may thus serve to ensure an adequate cytoplasmic supply of the karyophilic Vpr protein for incorporation into virions via its interplay with the p6 component of the Gag precursor during PIC assembly. Likewise, the presence of an export signal in Vpr may facilitate the export of p55^{gag} for the production of new virions. Indeed, the presence of p55^{gag} alters the localization of transfected Vpr (35). Whether the export function of Vpr influences cell cycle-arresting capabilities has yet to be determined. In summary, we have identified two novel import signals within HIV-1 Vpr and established the existence of a CRM1-dependent NES. The presence of these various import and export signals probably ensures representation of Vpr in the two different cellular compartments, where it performs critical functions in the viral life cycle.

ACKNOWLEDGMENTS

We thank Minoru Yoshida (University of Tokyo) for providing LMB, Harrison Lin for scientific contributions, and Thomas Hope (Salk Institute) for engaging discussions.

This work was supported by the UCSF-GIVI Center for AIDS Research grant NIH P30 MH59037.

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